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*Cell Injury, Repair, Aging and Apoptosis*

# Granulysin Produced by Uterine Natural Killer Cells Induces Apoptosis of Extravillous Trophoblasts in Spontaneous Abortion

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**Immune changes are known to occur in recurrent spontaneous abortion, but it is unclear whether either maternal natural killer (NK) cells or T cells attack fetus-derived trophoblasts. To clarify the immunological causes of spontaneous abortion, we examined the relationship between cytotoxic granule proteins in decidual lymphocytes, such as granulysin, granzyme B, and perforin, and the induction of apoptosis in extravillous trophoblasts (EVTs). The number of granulysin-positive CD56$^{bright}$ NK cells increased significantly in the decidua basalis during spontaneous abortion compared with normal pregnancy; however, granzyme B- and perforin-positive cells did not change. Interestingly, the expression of granulysin was also detected in the nuclei of EVTs in spontaneous abortion samples. When IL-2-stimulated CD56$^{bright}$ NK cells were cocultured with EVT cells (HTR-8/SV40neo), granulysin was found initially in the cytoplasm and then accumulated in the nuclei of the HTR-8/SV40neo cells. Furthermore, transfected cells expressing a GFP-granulysin fusion protein induced apoptosis in HTR-8/SV40neo cells independently of caspases. Our results suggest that granulysin-positive uterine NK cells attack EVTs; subsequently, the uNK-derived granulysin actively accumulates in the nuclei of EVTs, causing the death of EVTs due to apoptosis. These data support a new apoptosis pathway for trophoblasts via uNK-derived granulysin, suggesting that granulysin is involved in spontaneous abortion.** ***(Am J Pathol 2008, 173:653–664; DOI: 10.2353/ajpath.2008.071169)***

Apoptosis in trophoblasts is well known to be involved in human spontaneous abortion, intrauterine growth restriction, and preeclampsia.[1,2] However, direct evidence that cytotoxic T cells or natural killer (NK) cells cause apoptosis in extravillous trophoblasts (EVTs) has not been reported in humans, and little is known about the precise mechanism of apoptosis in EVTs of spontaneous abortion. Namba et al showed that interleukin (IL)-2R$\beta$ (CD122)-overexpressing transgenic mice showed 100% viable fetuses at gestational day 8 but 100% fetal death at gestational day 12, and proposed that excessive activation of uterine NK (uNK) cells can cause abortion.[3] In these mice, uNK cells were abnormally found within the placenta and the trophoblast layers had lost continuity.

Decidualization of the human endometrium following embryo implantation is normally associated with massive recruitment of CD16$^{-}$CD56$^{bright}$ NK cells. In early pregnant decidua, CD16$^{-}$CD56$^{bright}$ NK cells constitute the major immune cell population accounting for more than 70% among lymphocytes, whereas CD4$^{+}$ T cells and CD8$^{+}$ T cells are a minor population (<5%).[4,5] NK cells interact with target cells by a series of inhibitory and activating NK cell receptors constitutively expressed by uNK cells. Furthermore, CD16$^{-}$CD56$^{bright}$ NK cells recognize the major histocompatibility complex class I molecules HLA-C, -E, and -G via these inhibitory and activating receptors.[6] Once balance is lost, excessive activation signals may induce trophoblast lysis by these activating NK cells.[2,7] Hyperactivated NK cells release cytolytic granules such as granzymes and granulysin through perforin-induced pores. The distribution of perforin-positive uNK cells is essentially the same between

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normal and abortion model mice,[8] although a slight elevation of perforin-positive uNK cells in human sporadic miscarriage with a normal fetal chromosomal karyotype has been reported.[9] On the other hand, the distribution of granulysin-positive lymphocytes in normal pregnancy and spontaneous abortion cases has not been reported.

Granulysin is a novel cationic molecule present in the granules of cytotoxic T cells and NK cells.[10–12] Two molecular forms of granulysin result from post-translational cleavage at both the amino and carboxyl termini. The protein is synthesized as a 15-kd precursor form, which is sorted to the cytolytic granules where it is processed into a 9-kd effector form.[10,13] Granulysin exhibits potent cytotoxic activity against a broad panel of microbial targets, including tumor cells, transplant cells, bacteria, fungi, and parasites,[11–15] damaging negatively charged cell membranes because of its positive charge.[16] Granulysin is coexpressed with and functionally related to both perforin, a pore-forming protein related to the membrane attack complex of the complement, but structurally distinct, and granzymes, serine esterases that induce apoptosis by activating caspases.[17] Thus, granulysin plays important roles in the host defense against pathogens, and also induces apoptosis of the target cells in a mechanism involving caspases and other pathways.[16,18]

There is, so far, no report about the relationship between apoptosis of trophoblasts and uterine NK cells including granulysin in spontaneous abortion. We hypothesized that uNK cells contact and lyse EVTs, which detach from cytotrophoblast cell column by the release of cytotoxic granule proteins in decidua. In this study, we show that uNK cells induce apoptosis of EVTs in a granulysin-dependent manner in spontaneous abortion cases.

## Materials and Methods

### Tissue Collection

All samples for this study were approved by University of Toyama Ethics Committee, and informed consent was obtained from all patients. Ten specimens from elective termination of pregnancy (maternal age median 29 years, range 21–35 years; gestational age median 8 weeks, range 6–10 weeks) were obtained. These specimens were treated as normal pregnant subjects. Gestational age was calculated from the last menstrual period and confirmed by ultrasound measurements of crown-rump length. Twenty samples from first-trimester spontaneous abortion (maternal age median 30 years, range, 20–41 years; gestational age median 8 weeks, range 6–11 weeks) were collected. Anembryonic pregnancies or fetal death was confirmed by ultrasonography. All samples were collected by vaginal curettage; in normal pregnancy and spontaneous abortion, curettage was carried out within 24 hours after diagnosis. Both groups received the same exclusionary criteria: women receiving any medication or with infectious, autoimmune, or other systemic or local diseases were excluded. Clinical details were recorded for each woman (Table 1). Karyotype analysis was not performed in the spontaneous abortive specimens. The tissue samples were fixed in formalin and embedded in paraffin blocks for histological examination and immunohistochemical staining.

**Table 1** Comparison of Clinical Data from Patients with Spontaneous Abortions and Controls

| | Normal control (n = 10) | Spontaneous abortion (n = 20) |
|---|---|---|
| Age (year) | 29 (21–35) | 30 (20–41) |
| Gravidity[a] | 1 (0–4) | 1 (0–3) |
| PH: spontaneous abortion[b] | n = 1 | n = 2 |
| Gestational weeks | 8 (6–10) | 8 (6–11) |

Data are expressed as median (range).
[a]Abotrion times and live birth times.
[b]Numbers of patients with spontaneous abortion in past history (PH), excluding the abortion of this study.

### Isolation of Decidual Lymphocytes

Samples of decidua from different patients were not mixed to avoid the induction of allogenic reaction of leukocytes. For isolation of decidual cells, specimens from decidua of spontaneous abortion or normal pregnancy (gestational age, 6–10 weeks) were dissected free of products of conception and washed twice in phosphate-buffered saline (PBS). The total decidua tissue (4–9 g) was then minced into fragments of $\sim$1 $mm^3$ and digested for 20 minutes at 37°C under slight agitation in Dulbecco's modified Eagle's medium containing 0.125% trypsin (Sigma), 4.2 mmol/L $MgSO_4$, 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 50 Kunitz U/ml deoxyribonuclease type IV (Sigma). The cell suspension obtained was filtered through sterile stainless steel 50-$\mu$m wire mesh and washed once in PBS. The decidual mononuclear cells (leukocytes) were purified by the standard Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway) density gradient centrifugation after homogenization and filtration through a 32-mm nylon mesh as previously reported.[4] The cells were then suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin, and incubated for 2 hours at 37°C in an atmosphere of 5% $CO_2$ to allow adherent cells to attach to the plastic. The supernatant containing decidual lymphocytes was then collected and the cells were used for analysis.

### Cell Lines and Transfection

HTR-8/SV40neo cells (a kind gift from Dr. Charles H. Graham, Department of Anatomy and Cell Biology, Queen's University, Ontario, Canada), an EVT cell line, JEG-3 and BeWo, choriocarcinoma cell lines, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. JAR choriocarcinoma cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. For transient expression of GFP-fused protein, 3- to 7 $\times$ $10^5$ cells were inoculated into a 30-mm glass

base dish (Asahi Techno Glass, Tokyo, Japan) or a six-well plate (Becton Dickinson, San Jose, CA) a day before transfection. HTR-8/SV40neo cells were transfected with 1 $\mu$g of each plasmid DNA using FuGENE 6 reagent (Roche Diagnostics, Basel, Switzerland) and cultured for the times indicated. On the other hand, transient transfection of JAR and JEG-3 cells was performed with Lipofectamine LTX reagent (Invitrogen Corp., Carlsbad, CA). After that, the cells were analyzed by flow cytometry or immunocytochemistry.

For the assay of granulysin localization, we counted the number of the cells in which GFP completely merged with Hoechst 33342 nuclear staining as nuclear-localized cells, and the other cells as nuclear and cytoplasm-localized cells, respectively, in 100 GFP-positive cells. Each experiment was performed at least three times.

### *Plasmid Construction*

A GFP-expression vector, GFP-granulysin, was previously constructed.[19] Additionally, to construct GFPx2-fused proteins, cDNAs for a 9-kd granulysin corresponding to amino acid sequence $G^{63}$ through $R^{136}$ of the full-length granulysin were amplified by reverse transcription-polymerase chain reaction with Pfu polymerase (Stratagene) using total mRNA from normal peripheral blood mononuclear cells as a template. An expression vector, which expresses tandemly arranged GFP, was kindly provided by Dr. Naoko Imamoto (Discovery Research Institute, RIKEN, Saitama, Japan). The PCR product was cloned into the *Hin*dIII/*Bam*HI sites at the C′ ends of GFP (Figure 1).

### *Immunohistochemistry*

Five-micron sections from formalin-fixed, paraffin-embedded human tissues were deparaffinized in xylene and rehydrated in a graded series of alcohol followed by antigen retrieval by boiling in citrate buffer at 121°C for 15 minutes in an autoclave. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in methanol for 15 minutes, and nonspecific binding was blocked by incubating the sections in 5% normal goat serum. After extensive washes with PBS, the sections were reacted with a biotin-labeled anti-granulysin mouse monoclonal antibody (mAb) (1:100, RC8)[20] and mouse monoclonal antibody anti-CD56 (NCAM) (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). Further processing of the sections for the detection was performed according to the manufacturer's instructions provided with the Vectastain kit (Vector Laboratories, Burlingame, CA) or Vector blue alkaline phosphatase substrate kit (Vector Laboratories). After washing, sections were counterstained with Mayer's hematoxylin, washed in water, and successively immersed in graded ethanol solutions and xylene before coverslipping. In control sections, the primary antibody was replaced by control nonimmune mouse IgG (Vector Laboratories). Other first antibodies were used as follows: anti-perforin mAb (1:200, Thermo Scientific, Fremont, CA), anti-granzyme B mAb (1:200, Santa Cruz Biotechnology) and anti-cytokeratin mAb (AE1/AE2, 1:250, DAKO, Carpinteria, CA). When counting the percentage of positive cells in tissues, at least five high power fields were chosen randomly. Confocal images of fluorescent materials in spontaneous abortive tissues were collected using a confocal laser scanning microscope model TCS-SP5 (Leica Microsystems, Wetzler, Germany). For immunohistochemical fluorescent samples, the Alexa Fluor 488 goat anti-mouse IgG antibody and the streptavidin, Alexa Fluor 594 conjugate antibody, were used as secondary antibodies (Molecular Probes Inc., Eugene, OR).

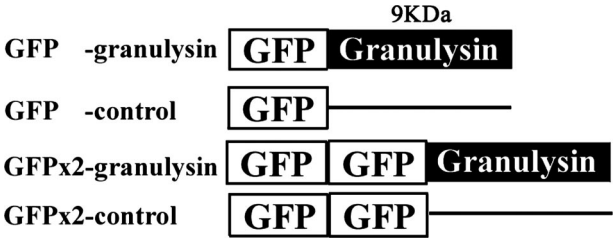



**Figure 1.** Structure of granulysin expression vectors. The structures of GFP-fused granulysin expression vectors are shown.

### *Immunocytochemistry*

The cells were fixed at 4% paraformaldehyde-PBS for 15 minutes and labeled with the first antibody, biotin-labeled anti-granulysin antibody (RC8) or anti-cleaved cytokeratin 18 mAb at dilution of 1:100. Negative controls were performed by replacement of the primary antibody with normal mouse serum. They were secondarily stained with the Alexa Fluor 594-streptavidin or the Alexa Fluor 594-conjugated anti-mouse IgG (Molecular Probes), respectively. They were finally stained with Hoechst 33342 (H33342) (Sigma-Aldrich, St. Louis, MO) for 10 minutes, washed with PBS, and observed under an All-in-One type fluorescence microscope BZ-8000 (KEYENCE, Osaka, Japan).

### *Purification of $CD56^+$ Cells, Transwell Experiments, and Coculture of HTR-8/SV40neo with Decidual Lymphocytes*

Isolated decidual lymphocytes were stimulated with or without 1 ng/ml IL-2 for 24 hours and then were gently washed with PBS three times. A small portion of the cells was fixed on a plate to check granulysin expression. For the isolation of $CD56^+$ lymphocytes, decidual lymphocytes were incubated with anti-CD56 mAb (Becton Dickinson) at 4°C for 20 minutes and then incubated with magnetic anti-mouse IgG beads. $CD56^+$ cells were separated using a magnetic cell sorting column. Flow cytometric analysis revealed that the purity of $CD56^+$ cells was >95%. These cells ($1 \times 10^7$) were added directly to $1 \times 10^6$ HTR-8/SV40neo for 24 hours. Subsequently, these cells were washed with PBS three times to remove the decidual lymphocytes and then were observed after fixation. Alternatively, to inhibit cell-cell contact, decidual lymphocytes were placed in a 0.2-$\mu$m Anopore membrane Nunc culture insert (Nalge Nunc International) and

cocultured with HTR-8/SV40neo in the lower chamber. Concanamycin A (Sigma-Aldrich), an inhibitor of perforin, was used in cell culture to inhibit the cytotoxicity of perforin at working concentration of 50 nmol/L.

### *Detection of Apoptotic Cells*

To assess apoptosis in spontaneous abortive tissue, a fluorescence terminal deoxynucleotidyl transferase dUTP nick-end labeling assay was performed according to the manufacturer's instructions (Apoptag *in situ* apoptosis detection kit; Oncor, Gaithersburg, MD). In brief, deparaffinized, dewaxed, and rehydrated sections were pretreated with 20 $\mu$g/ml proteinase K (Sigma-Aldrich) in 10 mmol/L Tris-HCl for 15 minutes, blocked with 10% normal goat serum, and then stained for terminal deoxynucleotidyl transferase dUTP nick-end labeling using a reaction mixture containing fluorescein-dUTP. Negative controls consisted of sections incubated without terminal deoxynucleotidyl transferase. Single immunohistochemical labeling using monoclonal antibodies against cleaved cytokeratin 18, M30 CytoDeath (Roche Diagnostics, Basel, Switzerland), after caspase-mediated cleavage, was performed according to the instructions provided with the Vectastain Elite ABC kit (Vector Laboratories).

### *Flow Cytometry*

One hundred microliters of a suspension of $1 \times 10^6$/ml of the decidual lymphocytes in PBS was first incubated with anti-CD56 PE (Becton Dickinson, San Jose, CA), permeabilized by incubating for 10 minutes with permeabilizing solution buffer (Becton Dickinson, dilute 10X solution 1:10 in deionized water), and then stained with biotin-labeled anti-granulysin mAb for 30 minutes at 4°C in the dark. Cells were washed and secondarily stained with fluorescein isothiocyanate-conjugated streptavidin (Becton Dickinson) for 30 minutes at 4°C in the dark. Cells were then washed, suspended in 500 $\mu$l of PBS, and immediately analyzed in a fluorescence-activated cell sorting (FACS) Callibur flow cytometer using the CellQuest program (Becton Dickinson). Other combination of antibodies was as follows: anti-CD3-PE (Becton Dickinson) and biotin-labeled anti-granulysin. We counted 15,000 cells in decidual lymphocytes.

For annexin V staining, HTR-8/SV40 cells transfected with GFP-control or GFP-granulysin vector were collected at 24 or 48 hours after transfection. The cells were then incubated with Alexa Fluor 594-conjugated annexin V (Molecular Probes, Eugene, OR) in an annexin V binding buffer (MBL Co. Nagoya, Japan) for 15 minutes at room temperature. Cells were washed, suspended in 500 $\mu$l of PBS, and immediately analyzed in a FACS Callibur flow cytometer using the CellQuest program. We counted the number of annexin-V positive cells in 20,000 GFP-positive cells.

### *Statistical Analysis*

The Mann-Whitney *U* test was used for comparisons between two groups. Correlations were tested by single regression analyses. Values of $P < 0.05$ were considered statistically significant using Statview.

## *Results*

### *Accumulation of Granulysin-Positive Cells in Decidua Basalis from Spontaneous Abortions*

We first examined the expression of granule proteins such as granulysin, granzyme B, and perforin on spontaneous abortion tissues by immunohistochemistry. Immunohistochemistry for granulysin was seen in the decidua basalis, the region of implantation with the fertilized ovum, but was scant in the decidua parietalis, the region removed from the implantation site (Figure 2A, a and b). In spontaneous abortion, granulysin-positive lymphocytes were detected in not only the decidua basalis but also in the decidua parietalis (Figure 2A, c and d). Additionally, the majority of granulysin-positive cells were detected in cytotrophoblast and cell column, and a few in syncytiotrophoblast in spontaneous abortion. Confocal microscopic images also showed similar results in the granulysin expression on the decidua basalis in normal pregnancy and spontaneous abortion (Figure 2B, right panels). As shown in Figure 2C, the numbers of granulysin-positive cells in the spontaneous abortive tissues (decidua parietalis: 54.4 ± 14.4/HPF, median 53.5, range 37–70; decidua basalis: 216.9 ± 83.4/HPF, median 227.5, range 135–300) were significantly higher than those in normal pregnancy (decidua parietalis: 5.5 ± 3.7/HPF, median 5, range 2–10; decidua basalis: 37.8 ± 9.1/HPF, median 36.5, range 25–50) ($P = 0.002$ and $P = 0.0076$, respectively). On the other hand, there were no significant differences in the number of perforin-positive or granzyme B-positive cells either in the decidua parietalis or decidua basalis between spontaneous abortion and normal pregnancy (Figure 2C). These results showed that granulysin-positive cells, but not perforin or granzyme B-positive cells, accumulate at implantation sites in spontaneous abortion cases.

### *Granulysin-Expressing Decidual Lymphocytes Are CD 56$^{bright}$ NK Cells*

The accumulation of granulysin-positive cells in the decidua basalis was verified by immunohistochemistry, but it is still unclear which cells contained the granule protein in the decidua. On the assumption that decidual lymphocytes consisting mainly of NK cells and T cells contain granule proteins, we checked the expression of granulysin in decidual lymphocytes obtained from spontaneous abortion cases by immunohistochemistry or flow cytometry. Expression of granulysin was detected mainly in the cytoplasm of CD56-positive cells (Figure 3A). After exchanging additionally the first antibody for the second antibody to rule out the possibility of nonspecific binding, we obtained the same results (data not shown). We further examined the expression of granulysin in uterine CD56$^{bright}$ NK cells or CD3$^+$ T cells of decidual lympho-

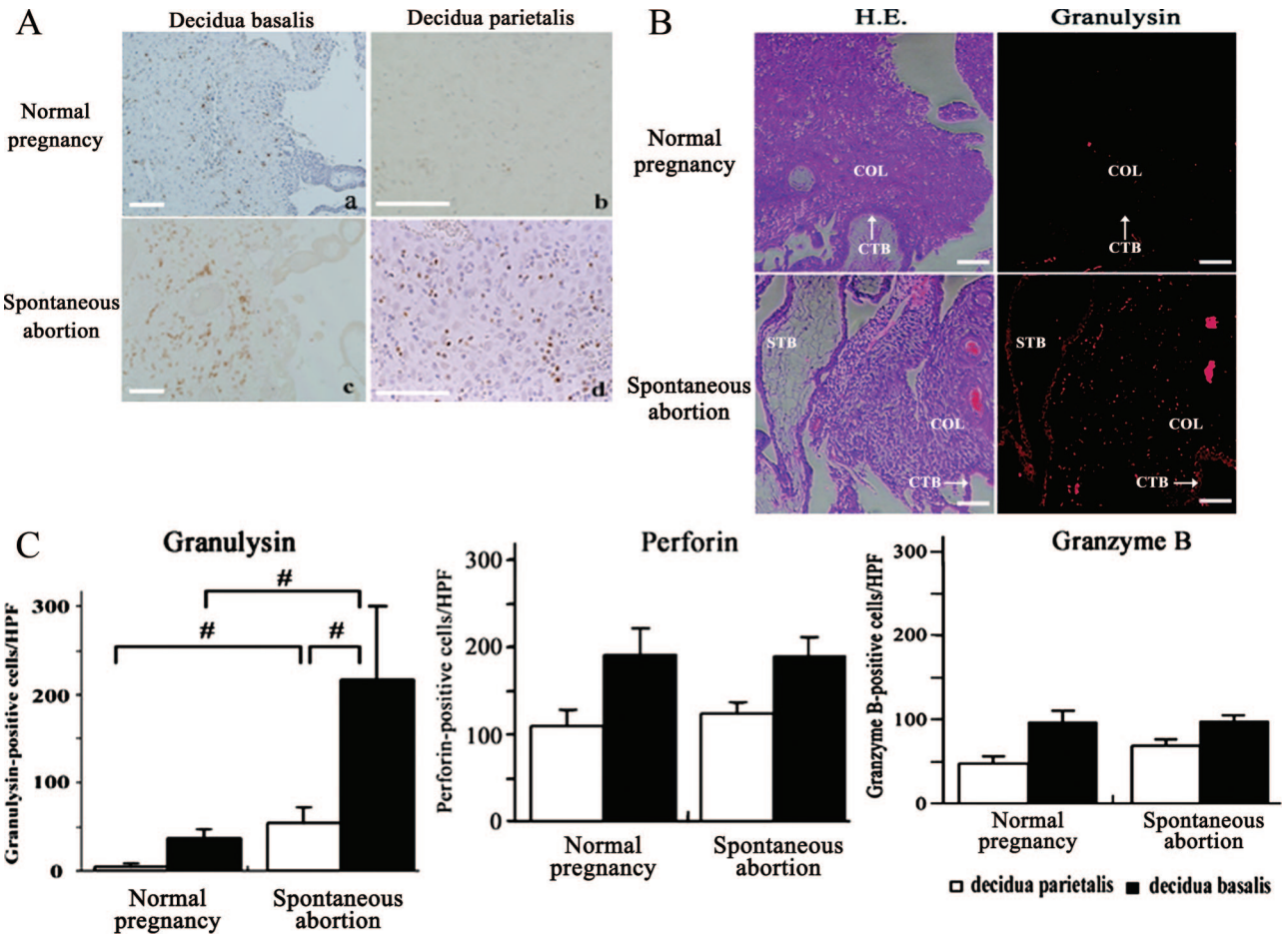



**Figure 2.** Expression of granule proteins in the decidua basalis and parietalis *in vivo*. **A:** Immunohistochemical studies showed expression of granulysin in the decidua basalis (**a**) and parietalis (**b**) in normal pregnancy, and decidua basalis (**c**) and parietalis (**d**) in spontaneous abortion at 8 weeks of gestation. Scale bars = 100$\mu$m. **B:** Serial paraffin sections of the maternal-fetal interface were stained with H&E (left panels) and anti-granulysin (right panels) in normal pregnancy (upper panel) and spontaneous abortion (lower panels) of 8 weeks' gestation. Right panels were confocal microscopic images. Scale bars = 100 $\mu$m. **C: N**umber of granulysin-positive cells (**left**), perforin-positive cells (**middle**), or granzyme B-positive cells (**right**). Two pairs of bars indicate the number of granule protein-positive cells in the decidua parietalis (white) and deciduas basalis (black) in normal pregnancy (left side, $n = 10$, gestational age 6–10 weeks) and in spontaneous abortion (right side, $n = 20$, gestational age 6–11 weeks) ($\#P < 0.05$). Data are the means and standard deviations of 20 experiments. STB, syncytiotrophoblasts; CTB, cytotrophoblasts; COL, the proximal site of the cell column.

cytes from normal pregnancy or spontaneous abortion cases by flow cytometry. Granulysin expression was mainly detected in CD56$^{bright}$ NK cells, but in only a few in CD3$^+$ T cells in spontaneous abortion cases (Figure 3B). The percentages of granulysin-positive cells in CD56$^{bright}$ NK cells were 20.6 ± 1.3% and 33.5 ± 4.2% in normal pregnancy and spontaneous abortion, respectively ($P < 0.05$). The percentage of granulysin-positive CD56$^{bright}$ NK cells in spontaneous abortion was significantly higher than that of normal pregnancy ($P < 0.05$), and that of CD56$^{bright}$ NK cells were significantly higher than that of CD3 T cells both in normal pregnancy and spontaneous abortion ($P < 0.05$). There was, however, no difference in the percentage of granulysin-positive T cells between normal pregnancy and spontaneous abortion (Figure 3C). These results indicated that CD 56$^{bright}$ NK cells, but not T cells, among decidual lymphocytes express granulysin in the decidua. Taken together, granulysin-positive CD 56$^{bright}$ NK cells increased in the decidua but also accumulated at the deciduas basalis in spontaneous abortion cases.

## *Granulysin Staining Observed in Extravillous Trophoblasts of Spontaneous Abortion Cases*

Several studies reported that apoptosis of EVTs was induced via activation of the tumor necrosis factor-$\alpha$ or Fas/FasL pathway in spontaneous abortion or preeclampsia.[1,21,22] In this context, the relationship between granulysin expression and apoptosis in EVTs was explored by immunohistochemistry. Interestingly, the expression of granulysin was detected in the nuclei of EVTs, which were reacted with cytokeratin antibodies, by diaminobenzidine staining (Figure 4A, a). The percentage of the granulysin-reactive cells was significantly increased in samples from spontaneous abortion compared with normal pregnancy (Figure 4B, 3.7 ± 0.9% versus 0.5 ± 0.5%, $P < 0.001$). To further examine the apoptosis of EVTs, we used two different methods: the terminal deoxynucleotidyl transferase dUTP nick-end labeling method and an antibody against cleaved cytokeratin-18, a detection marker in the early stage of apoptosis. Ter-

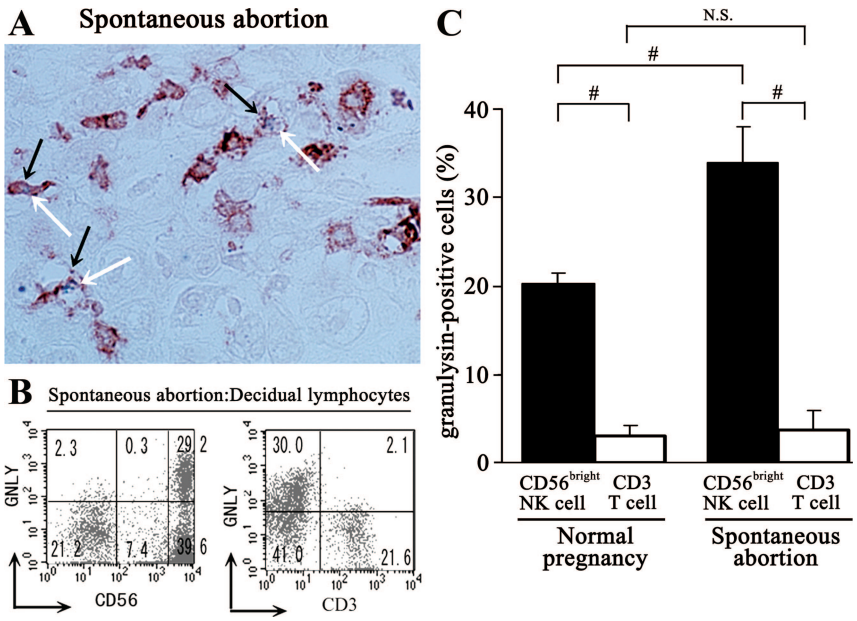



**Figure 3.** CD 56$^{bright}$ NK cells expressed granulysin in spontaneous abortion *in vivo*. **A:** Immunostaining tissue section of decidua of spontaneous abortion at 8 weeks of gestation revealed that granulysin (**white arrows**, blue dots) was detected only in some CD56-positive cells (**black arrows**, brown). **B:** Granulysin (GNLY) expression in decidual lymphocytes from a spontaneous abortion case was assessed by fluorescence-activated cell sorting analyses on CD56$^{bright}$ cells or CD3$^{+}$ cells. Data are from one representative experiment of three performed. The intensity of fluorescein isothiocyanate staining (GNLY-positive) is shown on the *y* axis, while the intensity of CD56 (left panel) or CD3 (right panel) staining is plotted on the *x* axis. The numbers represent the percentages of the dots in each gated area. **C:** The percentages of granulysin-positive cells in CD56$^{bright}$ NK cells (black bars) and CD3$^{+}$ cells (white bars) in normal pregnancy (left panels) and spontaneous abortion (right panels) were shown ($\#P < 0.05$). Data are the means and standard deviations of three independent experiments. N.S., not significant.

minal deoxynucleotidyl transferase dUTP nick-end labeling or cleaved cytokeratin-18 antibody reactive cells increased in spontaneous abortion cases, suggesting that these cells were dying due to apoptosis (Figure 4A, b and c). Furthermore, confocal studies clearly showed that some cytokeratin reactive EVTs invaded and detached from cytotrophoblast cell column in spontaneous abortion (Figure 4C, parts A and B). Granulysin expression was distinctly seen in both the cytoplasm and nuclei of these EVTs in spontaneous abortion (Figure 4C, parts C and D). On the other hand, granulysin expression was not detected in EVTs in normal pregnancy (Figure 4C, part E). Additionally, we next examined the granulysin mRNA expression in four choriocarcinoma cell lines, BeWo, JEG3 and JAR, and EVT cell line, HTR-8/SV40neo cells. These cells did not express granulysin mRNA (data not shown), suggesting that the granulysin in EVT cells was derived from the decidual NK cells. These results suggested that the increase in apoptotic EVT cells in spontaneous abortive tissue was related to the expression of granulysin in EVT cells by a mechanism in which uterine CD56$^{bright}$ NK cells transfer granulysin into EVTs.

## *Granulysin Produced by Uterine CD 56$^{bright}$ NK Cells Accumulated into Nuclei of HTR-8/SV40neo Cells* in vitro

We investigated the dynamic movement of native granulysin, which was derived from decidual lymphocytes, by an *in vitro* assay. In regard to this question, we checked the expression of granulysin on decidual lymphocytes obtained from normal pregnancy. Immunocytochemical staining showed that 1 ng/ml IL-2 enhanced granulysin expression in the cytoplasm of decidual lymphocytes after 24 hours of stimulation (Figure 5A). First, HTR-8/SV40neo cells were cultured with decidual lymphocytes with or without IL-2 stimulation. Spotted granulysin staining was found in HTR-8/SV40neo cells after 12 hours of coculture with IL-2-stimulated lymphocytes (Figure 5B). After an additional 6 hours, the expression level increased and the expression pattern was diffuse in the cytoplasm. Finally, 24 hours after coculture with decidual lymphocytes, a marked amount of granulysin staining was merged with nuclear staining (Figure 5C). On the other hand, granulysin staining was not detected in HTR-8/SV40neo with control. As shown in Figure 3B, CD 56$^{bright}$ NK cells mainly possessed granulysin in decidual lymphocytes. After the isolation of CD 56-positive cells from IL-2-stimulated decidual lymphocytes, these isolated CD56-positive lymphocytes were cocultured with HTR-8/SV40neo cells. The percentages of CD56-positive cells were increased from 60% to 95% in the decidual lymphocytes after the isolation (data not shown). Confocal microscopic study also showed the similar results. As shown in Figure 5A, the expression of granulysin in decidual lymphocytes was detected only in the cytoplasm but not nuclei. At 24 hours after the culture, granulysin was colocalized at some large nuclei in HTR-8/SVneo cells, which were treated with CD56-positive lymphocytes with IL-2 (Figure 5D, arrows). On the other hand, granulysin was detected at around small nuclei but not in nuclei in the control sample, which was cocultured with CD56-positive lymphocytes without IL-2 stimulation (Figure 5D, arrowheads), indicating CD56-positive lymphocytes without IL-2 stimulation did not secrete granulysin. These results suggested that hyperactivated decidual CD56-positive NK cells secreted granulysin accumulated in nuclei of HTR-8/SVneo cells. Furthermore, separating the IL-2-stimulated decidual lymphocytes and HTR-8/SV40neo with a Transwell culture system completely abrogated the expression of granulysin in HTR-8/SV40neo, suggesting that cell-cell contact was required for granulysin transfer from decidual lymphocytes (Figure 5E). When decidual lymphocytes were treated with IL-2 con-

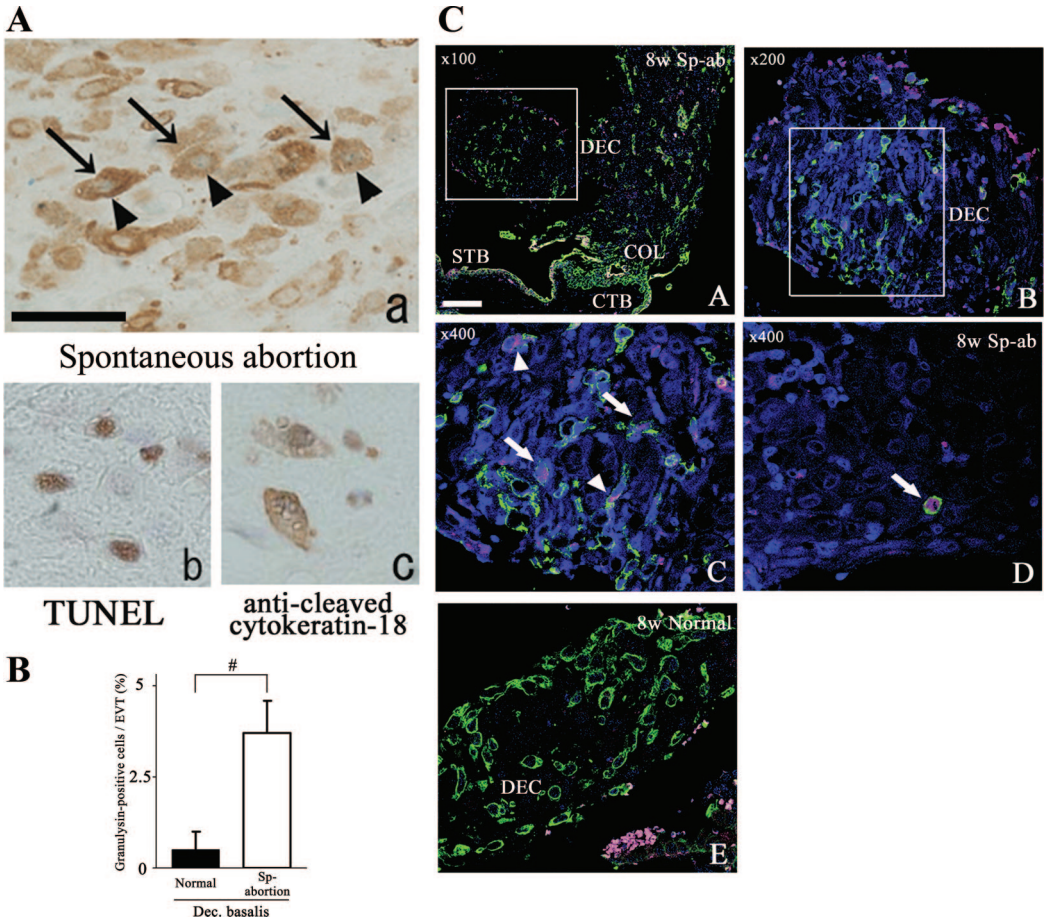



**Figure 4.** Extravillous trophoblast cells expressed granulysin in spontaneous abortion *in vivo*. **A:** Immunostaining tissue section of decidua of spontaneous abortion at 8 weeks of gestation revealed that granulysin (blue; **arrowheads**) was detected in cytokeratin-reactive cells (brown; **arrows**) in spontaneous abortion (**a**). Representative images of terminal deoxynucleotidyl transferase dUTP nick-end labeling staining (**b**) and anti-cleaved cytokeratin 18 staining (**c**) in spontaneous abortion case were shown. **B:** Quantitation of the granulysin-reactive cells in cytokeratin-reactive cells (EVTs) in the deciduas basalis from normal pregnancy (black, $n = 10$; gestational age, 6–10 weeks) and spontaneous abortion (white, $n = 20$; gestational age, 6–11 weeks). Cell counting was carried out on five randomized regions for each sample. Data are the means and standard deviations of 20 experiments ($\#P < 0.05$). **C:** Confocal microscopic images showed granulysin (red) and cytokeratin (green) staining in spontaneous abortion (**A–D**) and normal pregnancy (**E**) at 8 weeks of gestation. In **C**, expressions of granulysin were detected in the cytoplasm (**arrowheads**) and nuclei (**arrows**) in cytokeratin-reactive cells (green). **B** shows the region outlined by white line in **A**, and **C** is the region outlined in **B**. White bar, 100-$\mu$m scale bar; black bar, 50-$\mu$m scale bar. STB, syncytiotrophoblasts; CTB, cytotrophoblasts; COL, cell column; DEC, decidua basalis.

currently with concanamycin A, the inhibitor of perforin, before the coculture, concanamycin A treatment also blocked the expression of granulysin in HTR-8/SV40neo, suggesting that granulysin transfer is dependent on perforin expression (Figure 5F). These results suggested that CD $56^{bright}$ uNK cell-secreted native granulysin was transferred into the cytoplasm of EVTs in a cell-to-cell contact manner, resulting in the accumulation of granulysin into the nuclei of EVTs.

## *Apoptosis Is Induced by GFP-Fused Granulysin in Choriocarcinoma Cell Lines and HTR-8/SV40neo Cells*

We next examined whether transfer of granulysin into nuclei correlates with apoptosis of EVTs by using a GFP-fused granulysin expression vector (Figure 1). As shown in Figure 6A, GFP-fused granulysin, as well as native granulysin, transferred from cytoplasm into nuclei in a time-dependent manner. The percentages of cells, of which granulysin was detected exclusively in nuclei, were 27.7 ± 2.9, 46.7 ± 1.89, and 89.7 ± 1.27 at 12, 24, and 48 hours, respectively, after transfection in HTR-8/SV40neo (Figure 6B). On the other hand, the percentage of GFP expression in control was 13.2 ± 2.02 at 12 hours and the level of GFP expression in the control was stable for at least 48 hours after transfection. Consequently, to characterize the granulysin-induced cell death, the cells were stained with Hoechst 33342 (nuclear staining) and an anti-cleaved cytokeratin 18 antibody (an apoptotic marker of the early stage with spotting) after granulysin transfection. Spotted staining was detected in granulysin-positive cells on anti-cleaved cytokeratin 18 panels (Figure 6C, upper panels), whereas diffused staining was in control-positive cells (Figure 6C, lower panels), respectively. Furthermore, condensed nuclei were partially detected at 48 hours after transfection in granulysin-positive cells, suggesting an increase in apoptosis (Figure 6D).

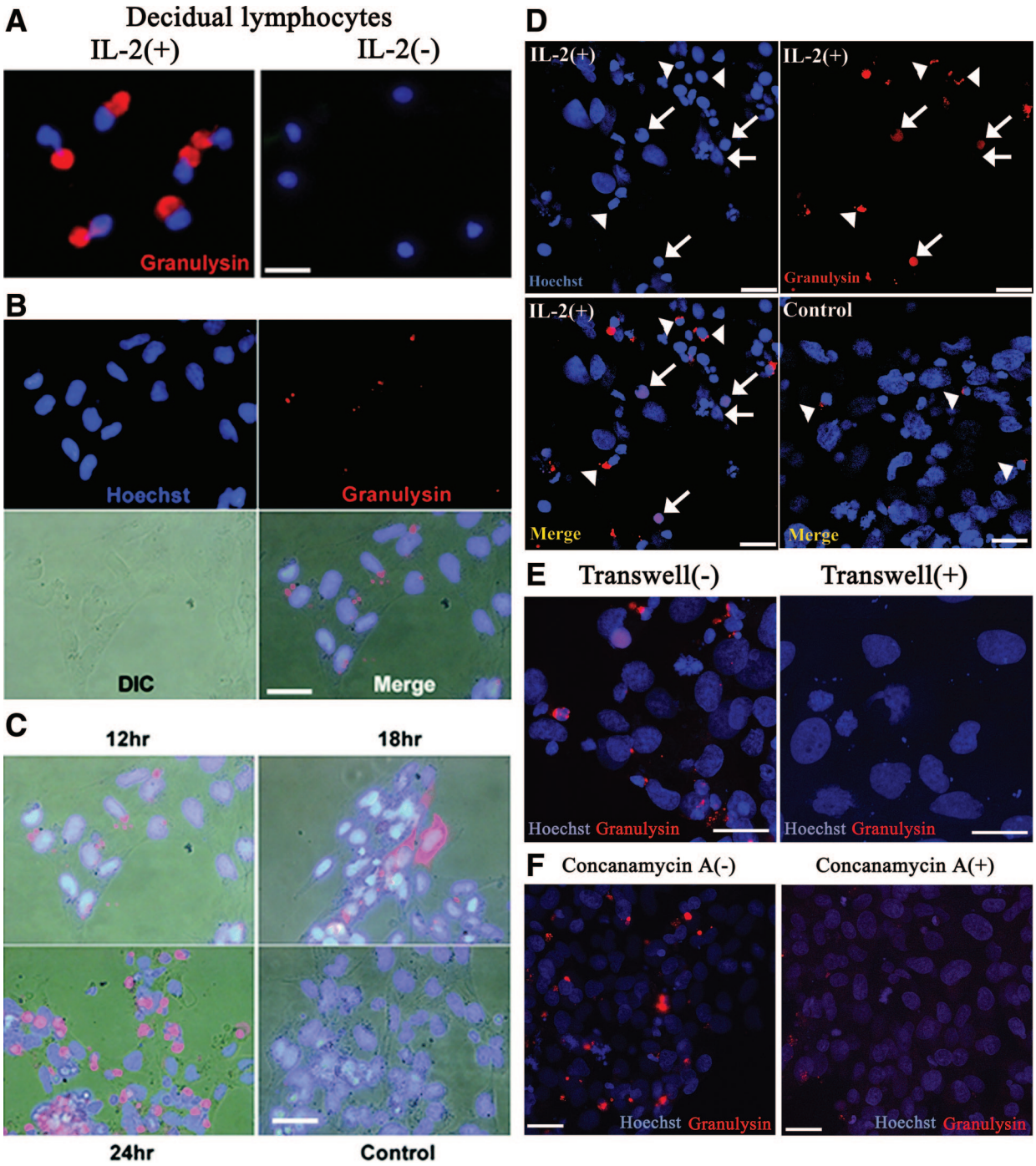



**Figure 5.** Localization of granulysin, derived from CD56$^+$ NK cells, in HTR-8/Svneo. Decidual lymphocytes, which were cultured with 1 ng/ml IL-2 for 24 hours, were cocultured with HTR-8/SV40neo for the indicated times. **A:** The reactivity of antibody against granulysin (red) on the decidual lymphocytes with IL-2 (left) for 24 hours was detected (blue: nuclear staining). Scale bar = 25 $\mu$m. **B:** HTR-8/SV40neo cells cocultured with decidual lymphocytes stimulated with IL-2 for 12 hours. Immunostaining of the samples showed that granulysin (red) was detected as dots in HTR-8/SV40neo. The right lower panel was the merged panel. Scale bar = 50 $\mu$m. **C:** These panels represented the localization of granulysin (red) in HTR-8/SV40neo at 12, 18, and 24 hours after coculture with IL-2-stimulated decidual lymphocytes. Scale bar = 50 $\mu$m. **D:** After the isolation of CD56-positive cells from IL-2-stimulated decidual lymphocytes, these CD56-positive cells were cocultured with HTR-8/SV40neo for 24 hours. Confocal microscopic images showed the colocalization (**white arrows**) of granulysin (red) and nuclear staining (blue) as well as the perinuclear localization (**arrowheads**) of granulysin. Control showed only the perinuclear localization (**arrowheads**) of granulysin. Scale bar = 25$\mu$m. **E:** HTR-8/SV40neo in the lower chamber was cocultured with IL-2-stimulated decidual lymphocytes, in the upper chamber with semipermeable Transwell membrane (right panel), or directly cocultured with IL-2-stimulated decidual lymphocytes (left panel) for 24 hours. Scale bar = 25 $\mu$m. **F:** HTR-8/SV40neo was directly cocultured with IL-2-stimulated decidual lymphocytes treated with (right panel) or without concanamycin A (left panel) for 24 hours. Both granulysin and nuclear staining are shown in red and blue, respectively. Scale bar = 25 $\mu$m.

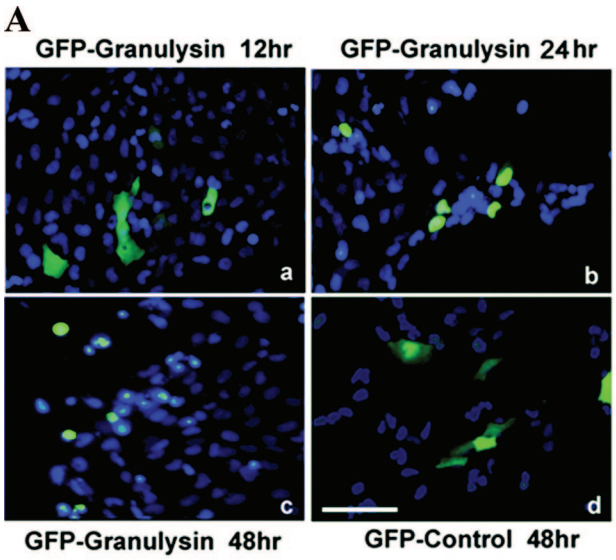
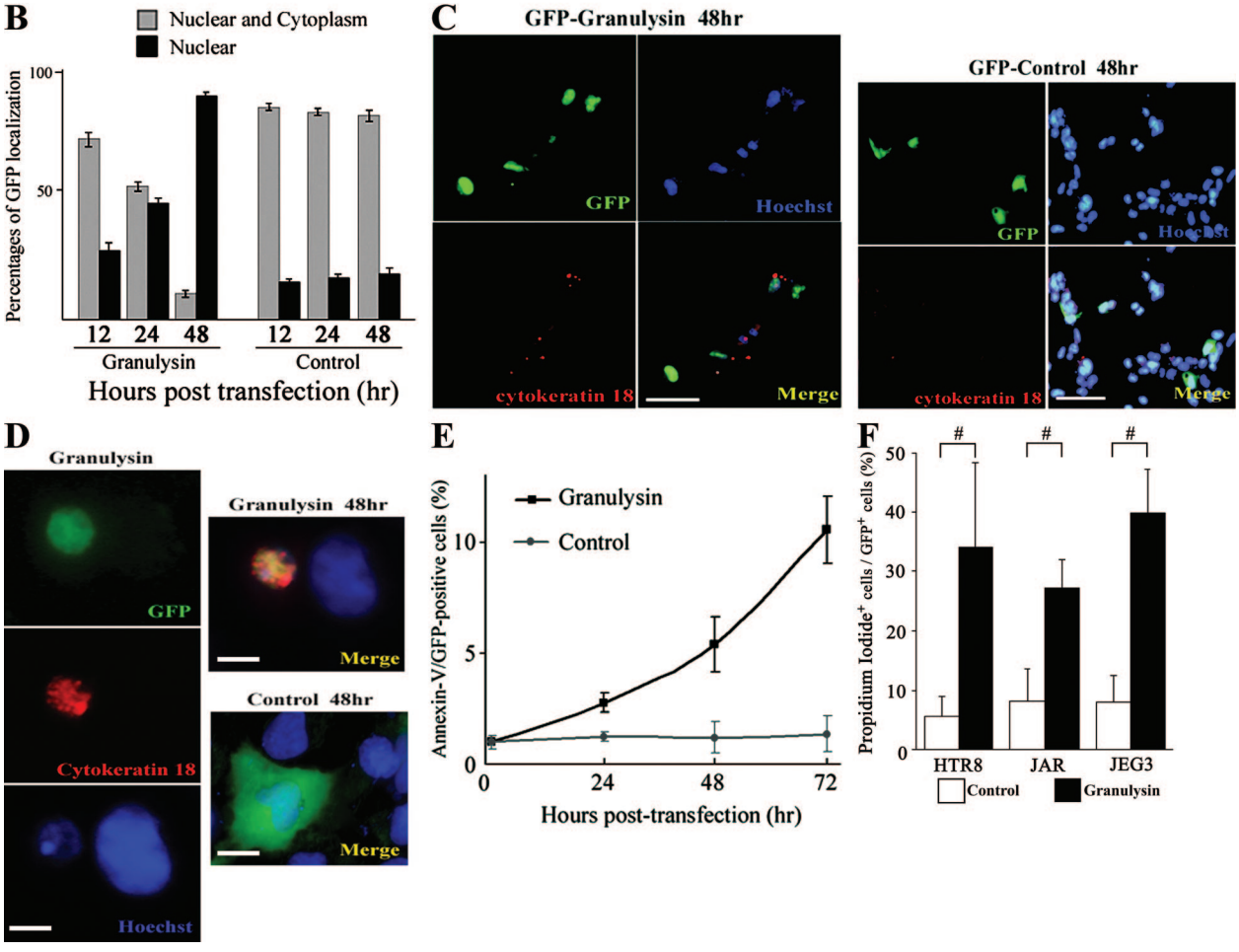



**Figure 6.** Granulysin-induced apoptosis of HTR-8/Svneo. **A:** Localization of GFP-fused granulysin in HTR-8/SVneo at 12(**a**), 24(**b**), and 48 hours (**c**; control shown in **d**). Scale bar = 100 $\mu$m. **B:** GFP localization in HTR-8/SV40neo after granulysin (**left**) or control (**right**) transfection. Gray bars indicated GFP localized in the nucleus and cytoplasm and black bars indicated GFP localized only in the nuclei. Cell counting was carried out on five randomized regions for each sample. **C:** GFP, nuclear staining (Hoechst), anti-cleaved cytokeratin 18 (red), and three merged panels (lower right panel) on HTR-8/SV40neo after granulysin (four left panels) and control (four right panels) transfection. The reactivity of antibody against cleaved cytokeratin 18 was detected as spotting, showing the early stage of apoptosis. Scale bar = 100 $\mu$m. **D:** GFP (green), anti-cleaved cytokeratin 18 (red), and nuclear staining (lowest) in the left three panels. The right panels showed three merged panels with granulysin (**upper**) and control (**lower**) transfection at 48 hours. Scale bar = 25 $\mu$m. **E:** Quantitation of the annexin-V reactive cells in GFP-positive cells at 24, 48, and 72 hours after granulysin (black line) or control (gray line) transfection. **F:** These graphs showed the percentages of propidium iodide-positive cells in GFP-positive cells at 24 hours after granulysin (black bars) or control (white bars) transfection in three cell lines ($\neq P < 0.05$). Data are the means and standard deviations of three independent experiments. GFP-granulysin transfection at 12h (a), 24h (b) and 48h (c). GFP-control at 48h (d).




Subsequently, the percentages of annexin V-positive cells among GFP-positive cells were estimated after granulysin transfection on HTR-8/SV40neo. The percentages of apoptotic cells were 2.7 ± 0.4, 5.7 ± 1.2, and 10.5 ± 1.5 at 24, 48, and 72 hours after transfection, respectively (Figure 6E). The kinetics of nuclear transport of granulysin coincided with the increase of apoptosis in EVTs after transfection. We further estimated the granulysin-induced cell death in choriocarcinoma cell lines, JEG3, and JAR cells as well as HTR-8/SV40neo, EVT cell line, by using propidium iodide. As shown in Figure 6F, cell death rates were significantly increased by granulysin transfection in all cell lines compared with control. Taken together, these results suggested that accumula-

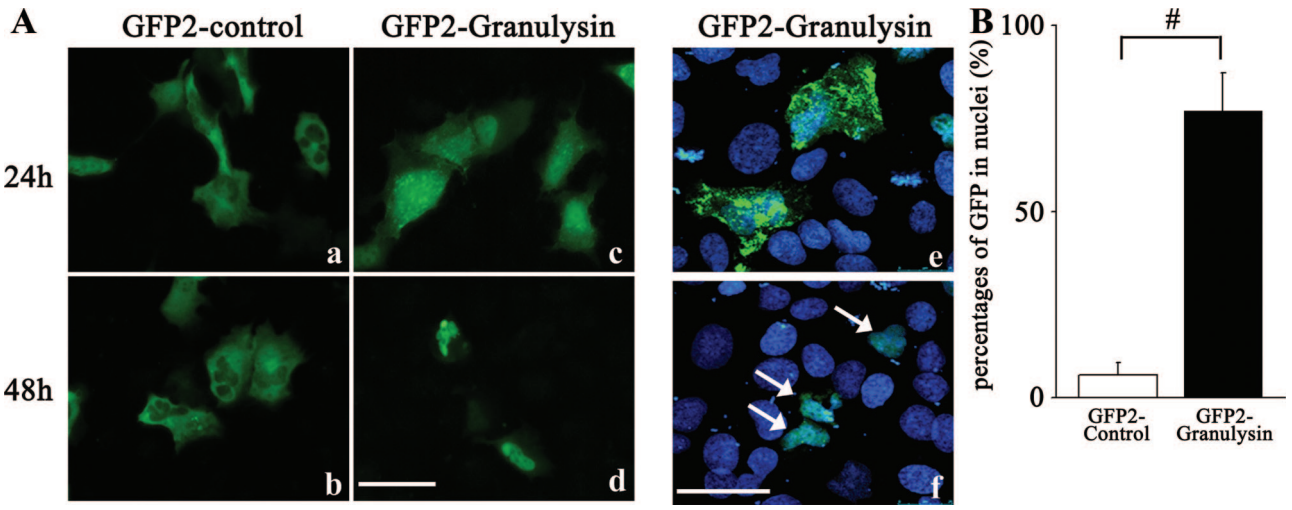



**Figure 7.** Nuclear accumulation of GFPx2-granulysin. **A:** Localization of the GFPx2-fused control (**a** and **b**) and granulysin (**c–f**) in HTR-8/SV40neo at 24 (**upper panels**) and 48 (**lower panels**) hours after transfection. These experiments were performed by both immunofluorescent microscopy (**a–d**) and confocal microscopy (**e** and **f**). Confocal microscopic images showed that GFPx2-fused granulysin (green, **arrows**) merged with Hoechst 33342 (blue) staining. GFPx2-control was detected only in peri-nuclei of HTR-8/SV40neo. Scale bar = 50 $\mu$m. **B:** GFP localization in HTR-8/SV40neo transfected with GFPx2-fused granulysin (black) or GFPx2-fused control (white) was evaluated. This graph showed the percentages of GFP localized only in the nuclei among GFP-positive cells. Cell counting was carried out on five randomized regions for each sample ($\#P < 0.05$). Data are the means and standard deviations of three independent experiments.

tion of granulysin into nuclei played important roles in the induction of apoptosis.

## *Active Accumulation of Granulysin into Nuclei on HTR8/SV40neo*

The molecular weight of granulysin is 9 kd, and the weight of GFP-fused granulysin is 36 kd. It is therefore possible that both granulysin and GFP-fused granulysin easily pass through nuclear pores, because diffusion of substances into nuclei partially relies on the molecular weight, which is under 40 kd. To exclude the possibility of spontaneous nuclear diffusion of granulysin, we constructed a new vector, pEGFPx2-granulysin, which links granulysin to GFP-cDNA at the C′ end (Figure 1). Conceptually, neither GFPx2-granulysin nor the GFPx2-control, tandemly arranged GFP, should migrate into nuclei in a simple diffusion manner. An immunocytochemical fluorescent study showed that green dots were detected in cytoplasm and nuclei on GFPx2-granulysin transfected cells at 24 hours after transfection and then accumulated into nuclei at 48 hours after transfection (Figure 7A, c and d). Confocal microscopic studies clearly showed the nuclear accumulation of granulysin in HTR-8/SV40neo (Figure 7A, e and f). On the other hand, the GFPx2-control was only detected in cytoplasm, but not the nuclei, at 48 hours as well as 24 hours after transfection (Figure 7A, a and b). The percentage of cells, of which GFPx2-granulysin was detected exclusively in nuclei, was 76.9 ± 10.4, while that of GFPx2-control in nuclei was 5.9 ± 3.5 at 48 hours after transfection (Figure 7B), indicating that the percentage of GFPx2-granulysin in nuclei was significantly higher than that of GFPx2-control. These results indicated that granulysin actively migrated into nuclei of HTR-8/SV40neo independently of simple diffusion.

## *Granulysin-Induced Apoptosis Is Independent of Caspases*

We finally explored the mechanism of granulysin-induced apoptosis by staining with propidium iodide, as a cell death marker, between granulysin and control transfection samples. As shown in Figure 6F, we obtained a significant difference in the percentages of propidium iodide-positive cells between granulysin and control transfection at 24 hours, and the percentages of propidium iodide-positive cells were 74.4 ± 4.7 and 18.3 ± 4.3 in granulysin and control at 48 hours after transfection in HTR-8/SV40neo (data not shown). Subsequently, we examined whether a general caspase inhibitor, z-VAD-FMK, inhibited granulysin-induced cell death in this experiment. No inhibition of cell death was observed on granulysin-transfected HTR-8/SV40neo cells, which were treated with z-VAD-FMK, after 48 hours. There were no effects on granulysin-induced cell death by treatment with the respective caspase inhibitors caspase-1, -3, -4, -6, -8, -9, -10 and -13 inhibitors, as well as a general caspase inhibitor (data not shown).

## *Discussion*

In pregnancy, an increase in trophoblast apoptosis may induce insufficient trophoblast invasion and cause pregnancy-related disorders such as spontaneous abortion, preeclampsia, intrauterine growth restriction, or preterm labor.[1,2,21–24] On the other hand, numerous studies have reported that the predominance of Th2 over Th1 cytokines plays some roles in a successful pregnancy.[25,26] Olivares et al previously reported that decidual lymphocytes from human spontaneous abortion cases induced

apoptosis in JEG-3 cells, a choriocarcinoma cell line, by interacting with the target cells and IL-2-stimulated decidual lymphocytes.[1,2,21–24] Predominant Th1 type immunity is present in recurrent spontaneous abortion,[25,26] and apoptosis of trophoblasts is higher in spontaneous abortion with Th1 type immunity.[7] Furthermore, we also reported that serum granulysin is a good marker for detecting Th1 type immunity.[27] However, it is still unclear whether maternal lymphocytes can kill fetus-derived trophoblasts in spontaneous abortion cases.

This study has three major findings. The first is that the number of granulysin-positive $CD56^{bright}$ uNK cells was significantly higher in the decidua basalis in spontaneous abortion than in normal pregnancy, while there was no difference in the numbers of perforin-positive and granzyme B-positive cells. The number of granulysin-positive cells was also increased in decidual lymphocytes from spontaneous abortion cases than normal pregnancy subjects. Taken together, these findings showed that granulysin-positive $CD56^{bright}$ uNK cells were increased and accumulated in the decidua basalis in spontaneous abortion, suggesting that granulysin may be a key substance for spontaneous abortion. The second finding is that apoptosis of EVTs correlated with the granulysin transfer from $CD56^{bright}$ uNK cells in spontaneous abortion cases in both *in vivo* and *in vitro* experiments. Finally, granulysin transfer is dependent on both perforin and cell-cell contact, and transferred granulysin actively accumulated into nuclei in EVT cell line. Considering all results, we speculate that the mechanism by which granulysin induces apoptosis of EVTs plays an important role in inducing spontaneous abortion. This is the first report that granulysin, which is produced by $CD56^{bright}$ uNK cells, is involved in the induction of apoptosis of EVTs in spontaneous abortion by *in vivo* and *in vitro* experiments.

Numerous reports have shown that $CD8^+$ cytotoxic T cells and NK cells kill target cells such as virus-infected cells, some pathogenic microorganisms, tumor cells and other host cells, to defend the host against granulysin.[10,28] Although it is still an unknown mechanism by which decidual lymphocytes induce apoptosis on EVTs, we gave a new insight that granulysin, a cytotoxic granule protein of NK cells, contributes to the apoptosis of EVT in spontaneous abortion cases. Our previous report showed that granulysin is associated with the development of preeclampsia as a Th1 marker; this study showed that granulysin plays important roles affecting the development of a disease, spontaneous abortion, as well as defending the host.[29]

Our system, using expression vectors, gave new findings on the dynamic state of granulysin within cells. We have already reported that GFP-granulysin induces the cell death with nuclear accumulation in HeLa cells independently of caspase.[19] In this study, GFPx2-granulysin, which existed diffusely in the cytoplasm, gradually accumulated into nuclei, whereas GFPx2-control stabilized in the cytoplasm, suggesting that granulysin actively accumulates in the nuclei. Additionally, we showed that granulysin may be able to induce apoptosis in EVTs. As the analysis of molecular sequences demonstrated that granulysin has no nuclear localization signal among the well-known nuclear localization signals, the mechanism of granulysin accumulation in nuclei is unknown. To date, some reports have shown that several factors such as sphingomyelinase, intracellular calcium concentration, cytochrome *c* release, and apoptosis-inducing factor, played important roles in granulysin-induced apoptosis by using other systems, through which granulysin permeated to the target cells using a medium containing recombinant granulysin.[16,30–32] To resolve the mechanism by which granulysin induces apoptosis of EVTs, we checked the correlation between apoptosis-related molecules and granulysin expression. Some caspase inhibitors had no effect on granulysin-induced cell death. Consequently, we hypothesized that mitochondria, which is attacked by granulysin, releases apoptosis-inducing factor to cytoplasm, and then apoptosis-inducing factor translocates to the nuclei of EVTs. However, Western blotting and confocal microscopic studies showed that the translocation of apoptosis-inducing factor did not change together with granulysin. Thus, uNK-derived granulysin may induce apoptosis of EVTs by itself. There are some possibilities that direct or indirect chromatin binding of granulysin may affect chromatin remodeling, resulting in the induction of apoptosis, but this is still unknown. Therefore, further studies are needed to verify this mechanism.

Straszewski-Chavez et al also showed down-regulation of X-linked inhibition of apoptosis (XIAP) renders first-trimester trophoblast cells sensitive to Fas-mediated apoptosis.[33] In this study, granulysin expression did not affect the expression of XIAP on HTR-8/SV40neo cells (data not shown). The decrease in XIAP induced the activation of caspase-8, -9 and -3,[33] but granulysin-induced apoptosis was independent of caspases in this study and a previous study.[19] EVTs inhibit caspase cascade activation by XIAP, because first trimester trophoblast cells express both Fas and FasL. In other words, XIAP may be an indispensable factor for physiological function, such as differentiation or invasion of trophoblasts.[33] Given that granulysin has an etiological factor, but not a physiological factor, it may effectively induce apoptosis in EVTs independently of caspases and XIAP.

Olivares et al and Kokawa et al have suggested that excessive destruction of trophoblasts by apoptosis results in spontaneous abortion.[2,21] In regard to this point, several apoptosis pathways of EVT are known. First, Reister et al reported that macrophages secrete tumor necrosis factor-$\alpha$ and tumor necrosis factor-$\alpha$ induces apoptosis of EVT.[23] Second, Mor et al showed that isolated first-trimester trophoblast cells can express Fas on their surface and the Fas ligand could induce apoptotic death of trophoblasts.[33] Considering all our results *in vivo* and *in vitro*, we propose a third pathway, the granulysin pathway, in the course of apoptosis of EVT by uNK cells.

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